STUDIES ON BIONOMICS AND PATHOGENICITY OF LUMINESCENT BACTERIA

DISSERTATION SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF FISHERIES SCIENCE
(MARICULTURE)

OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)

BY

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JULY 2000

Luminescent Bacteria- *Vibrio harveyi*
In Darkness


Dedicated
To My
Parents

CERTIFICATE

Certified that the dissertation entitled **"Studies on Bionomics and Pathogenicity of Luminescent Bacteria"** is a bonafide record of work done by **Mr. S. John Josephraj** under our guidance at the Central Marine Fisheries Research Institute during the tenure of his **M.F.Sc (Mariculture)** Programme of 1998-2000 and it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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I hereby declare that this dissertation entitled "**Studies on Bionomics and Pathogenicity of Luminescent Bacteria**" is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

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सारांश

मोनोडॉन व इंडिकस झींगों में *लूम्निसेन्ट बाक्टीरिया* से होनेवाला प्रभाव समझने केलिए बाक्टीरिया के जीवपारिस्थितिकी व रोगजनकता पर अध्ययन किया. निरीक्षण उपर्युक्त झींगा जातियों के डिंभक चक्र में किया था. समुद्र से पकडे गए झींगों के आँत्र में *लूम्निसेन्ट बाक्टीरिया* का विघटन तुलनात्मक अध्ययन केलिए किया था. मोनोडन झींगा के आँत्र में बाक्टीरिया का परास प्रति ग्राम में $30x10^2$ सी एफ यू -$3x10^4$ और इंडिकस झींगा के आँत्र में $2x10^2$ -$3x10^4$ सी एफ यू देखा गया जबकि हैचरी में पालित झींगा डिंभक में *लू. बाक्टीरिया* नहीं था. हैचरी में पालित डिंभक में बाक्टीरिया के अभाव का कारण अच्छा देखभाल माना जाता है, जबकि समीपस्थ समुद्री पानी में इस बाक्टीरिया का परास प्रति मि.ली. में $5x10^4$ सी एफ यू है . विघटन किए 13 स्ट्रेनों की जीव-रासायनिक स्थिति ऐसी थी कि ऑक्सिडेस और काटालेस एनज़ाइम की उत्पादकता शत प्रतिशत थी. अधिकांश विघटक अरागिनिन से निष्क्रिय था जबकि लैसीन और ऑरनिथैन का डीकार्बोक्सिलीकरण प्रतिशत यथाक्रम 61.53 और 38.5 था. सभी स्ट्रेनों ने ग्लूकोस, सुक्रोस ,सेल्लोबियोस का किण्वन किया पर लाक्टोस, अराबिनोस, इनोसिटोल का किण्वन नहीं हुआ. सभी बाक्टीरिया स्ट्रेन नमक पसंदी थे. 10% सोडियम क्लोरैड पानी में वृद्धि नहीं के बराबर थी.

आन्टिबयोटिक्स देने पर मल्टिपल ड्रग रेसिस्टन्स 92.3% दिखाया. बाक्टीरिया के 61.53% स्ट्रनों ने हीमोलैसिस दिखाया. मोनोडन और इंडिकस झिंगों के पश्च डिंभकों में *वी. हार्वेई* की रोगजनकता पर किए अध्ययन ने व्यक्त किया कि ये जीवन केलिए घातकारक हैं. *वी.स्प्लेन्डियस* जाति इससे भी घातक दिखायी पडी. मरणासन्न और मृत पश्च डिंभकों से लूम्निसेन्ट बाक्टीरियाओं का पुनर्पृथक्करण हो पाया जो यह प्रमाणित करता है कि ये *लूमिनोसिस* में होनेवाला वर्णहीनता का एजेंट है. झींगा हैचरियों में होनेवाली झींगा मृत्युता का कारक सिर्फ *लूम्निसेन्ट बाक्टीरिया* नहीं है , पर कई पर्यावरणिक घटकों से यह होता है . स्वस्थ झींगों का नियमित पालन उन्हें किसी प्रकार का स्ट्रेस दिए बिना करने पर *लूमिनोसिस* से डरने का प्रश्न नहीं उठता , हैचरियों से स्वस्थ झींगा बीजों का उत्पादन हो सकता है .

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude to my Supervising Guide **Dr. V. Chandrika,** Senior Scientist, FEMD, C.M.F.R.I., Cochin for her unflinching support, perpetual attention, guidance and encouragement throughout the period of my study as well as in the preparation of the manuscript. I am greatly indebted to members of advisory committee, **Dr. K.C. George,** Senior Scientist, PNPD and **Dr. A. Laxminarayana,** CTO, TTC for their guidance, supervision and encouragement throughout my work.

I am grateful to **Dr. V. Narayana Pillai,** Director, and **Dr.M.Devaraj,** former Director, C.M.F.R.I. for providing me the facilities to carry out my work at the Institute during the course of this study.

I am immensely thankful to **Dr. R.Paulraj,** Officer-in-Charge, PGPM for his timely help and encouragement throughout my study. I also wish to thank **Dr. D. Noble,** Senior Scientist, PNPD for his kind interest and help offered throughout my M.F.Sc course.

I would like to acknowledge my earnest thanks to Shri.G.S. Daniel Selvaraj, Senior Scientist and Dr. P. Kaladharan, Scientist for their kind help and encouragement.

I am very much thankful to the authorities of MPEDA hatchery, Vallarpadam for providing the samples during my study period.

I wish to express my gratitude to Ms.M.M. Latha (CSIR) and Ms. I.Sandhya Rani (CIFE) for their sincere help and encouragement.

I take this opportunity to thank all my classmates and juniors for their support and co-operation. I express my whole hearted thanks to Ms.Maya, Ms. Juliet, Yesu (CIFT) and Pauls for their help in few stages of my work.

I am very much obliged to Udya, Bisu, Sumanth, Radhika, N.Rudhramurthy, Sunil Lambeda, Manoj, Sathish Sahayak for their help and encouragement.

I wish to express my gratitude to Shri P.M. Aboobacker, Technical Officer, PGPM for his help.

My special thanks to Mr. Raju and Ms.Indhu Konica Square (Flash finish) for helping me in taking the photographs. I also thank Mrs.Sajitha for her skillful and prompt service done while typing my dissertation.

I acknowledge the Indian Council of Agricultural Research for awarding me with fellowship during the tenure of my post graduation.

Words cannot express my thanks to my parents, sister and loving brothers to whom this work is dedicated.

I express my reverence to the "Almighty" who has afford me enormous strength, during the present work and all my years of study.

John Josephraj.S.

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1. INTRODUCTION

INTRODUCTION

The development of intensive shrimp culture and the upgrading of traditional shrimp farm have created the need for more hatchery seeds. Though hatchery production of shrimp seeds was successful, there is every chance that the seeds will be affected by infectious and noninfectious diseases. The shrimp larvae are susceptible to bacterial diseases like bacillary necrosis, larval necrosis, larval luminosis and juvenile vibriosis and generally greatest problem is met in the warmest months (Karunasagar *et al.*,1994) The impact on the host is systemic infection of the soft tissues of larvae resulting in tissue luminosis and necrosis (due to production of exotoxin by bacteria) and death. Diagnostic techniques like histology can reveal tissue necrosis and the presence of rod-shaped bacteria (usually slightly curved) within the tissues (Lightner,1983). It is possible to reisolate the luminous strains in Seawater Complex (SWC) agar from the tissues of infected larvae (Ruby and Nealson,1978;Lavilla Pitogo *et al.*,1990)

Vibrios are obiquitous in seawater and apart from seawater, gravid female shrimps can discharge, abundant colonies of luminescent bacteria while captivity in hatchery (Baticodos,1988;Lavillo Pitogo *et al.*,1992) Hence eradication of the etiological agent is impossible. Luminosis appears to be directly related to poor husbandry(Jawahar Abraham *et*

al.,1997a) Sources of infection are broodstock, algal cultures and incoming sea water. Numerous strategies have been attempted for the control of luminosis in penaeid shrimp culture operations. These strategies have ranged from the use of improved husbandry practices to stocking "Specific Pathogen Free" (SPF) or "Specific Pathogen Resistant" (SPR) species of stocks. Currently available control strategies are based on **prevention by exclusion** (Moriarty,1998)

OBJECTIVES OF THE PRESENT STUDY:

To monitor a shrimp hatchery for the occurrence and distribution of Luminescent bacteria.

Quantitative and qualitative analysis of Luminescent bacteria in the wild shrimps.

To know the percentage composition of Luminescent bacterial population in the cochin waters.

To know the antibiotic sensitivity and drug resistance pattern of Luminescent bacteria.

To study the Virulence of the isolated strains.

2. REVIEW OF LITERATURE

REVIEW OF LITERATURE

Bioluminescence is a widespread phenomenon characterised by light emission produced in luciferase catalysed oxidation of the substrate, luciferin. Bioluminescence is produced by an incredible range of marine organisms from bacteria and single-celled protists to fish and squid. It has been reported that the various habitat of these bacteria include a variety of commensal, parasitic, saprophytic and free living niches and also the luminous organ of some fish species (Baumann and Baumann, 1977; Hastings and Nealson, 1977; Orndorff and Colwell, 1980).

Earlier workers assigned these luminous bacteria to a variety of genera. Beijerinck (1889) proposed that taking into account the following characters should place all luminous bacteria.

1. They all grow better when the food contains 3.5 % sea salt or isotonic ratios of other mineral salts.
2. They lose their (light-emitting) power by the addition of 2% or more glucose to their food, (because) they form then an acid and take on very peculiar shapes exhibiting pleomorphism.
3. Peptone is their main source for nitrogen intake; they receive their carbon from very dilute solution of glucose, levulose, maltose, galactose, calcium lactate, and above all from glycerol and this assimilation is accompanied by the production of light.

4. They develop in a neutral or slightly alkaline medium, and a trace of acid is sufficient to extinguish the light.

5. They never form spores; they can all be brought by culture to motile stage, which swim towards the sources of oxygen and under certain conditions taking on the shape of spirilla and of vibrios.

6. None of them secretes diastatic or invertase enzymes. Hence, soluble starch, cane sugar and milk sugar can neither serve for food not for the production of light, because these substances as such are not oxidized.

7. All give a continuous light spectrum between the D and G lines, hence, in the yellow, green and blue region.

According to Beijerinck (1889) the loss of luminescence is due to hereditary influence. Temporary loss of luminescence can be caused by modifying the media. Weakening in luminocity is always accompanied by arrest in growth, loss of gelatin liquefying power and pleomorphism. Even aldehyde such as lophine, amarine, hydrobenzamide, bitter almond oil, trimethylene oxide $(CH_20)_3$ and acetaldehyde in small amount causes the extinction of light. The ability of hydrogenperoxide to support luminescence was also explained by him by the violent decomposition of this compound under the influence of luminescent bacteria.

It was reported in 1916, by the same author that these microbes are closely related to choleras. After this much confusion was there in the nomenclature and taxonomy of luminous bacteria.

Hendrie *et al.*(1970) listed three genera based on the phenotypic studies of luminescent bacteria as *Vibrio, Photobacterium* and *Lucibacterium.* The genus *Lucibacterium* was created to accomodate species which were similar to *Vibrio* however possession of peritrichous as well as polar flagellation and insensitivity to 0/129 precluded the inclusion of these strains in the genus *Vibrio* (Hendrie *et al.*, 1970). The creation of the genus *Lucibacterium* failed to recognize the shift from polar flagellation in liquid medium to peritrichous like flagellation in solid medium which commonly occurs with marine *Vibrio* species. So this strains were transferred to the genus *Beneckea* (Reichelt *et al.*, 1976). At present this has been placed in genus *Vibrio* following the isolation of the genus *Beneckea* (Baumann *et al.*, 1980). He also reported that the genera *Photobacterium* and *Vibrio* can't be readily distinguished by phenotypic characterisation.The diagnostic characters of the luminous strains and their biological significance was discussed by Jayabalan and Ramamoorthi (1979).

Baumann and Baumann (1980); Bang *et al.* (1981) did extensive studies on the aminoacid sequence of the glutamine synthetase and superoxidase dismutase enzymes indicating evolutionary divergence between the genera *Photobacterium* and *Vibrio.* Based on the classification of Bergey's manual of systemic bacteriology, Vol. I (1984) Jawahar Abraham et *al.* (1999) reported under optimum condition, atleast one strain of the following bacteria emit atleast a feeble light.

Family: Vibrionaceae:

* *Alteromonas hanedai (Shewanella hanedai)*	-	only obligate aerobe emitting light; grows at 4°C not at 35°C.
* *Photobacterium leiognathi*	-	fish light organs; no growth at 4°C; growth at 35°C
* *Vibrio cholera*	-	a few, rare strains emit faint light; growth at 40°C.
* *Vibrio fischeri*	-	yellow-orange growth at 30°C; no growth at 4°C; emits light weakly.
* *Vibrio (Photobacterium) phosphoreum*	-	growth at 4°C, no growth at 35°C; brightest light emitter at 35°C all bacteria; isolated from fish light organs & seawater
* *Vibrio (Lucibacterium) harveyi*	-	many strains emit light
* *Vibrio logei*	-	growth at 4°C; most strains emit light
* *Vibrio (Beneckea) natriegens*	-	survives years at room temp; even drying
* *Vibrio splendidus*	-	most strains emit strong light; growth at 40°C

Family: Enterobacteriaceae:

* *Xenorhabdus luminescens* - isolated from,nematodes *(photorhabdus)*

Xenorhabdus (Photorhabdus) luminescens - is classified in the Enterobacteriaceae family and all the other luminous bacteria are placed in the closely related family vibrionaceae.

Vibrio harveyi is a marine form commonly found in the water column and on fish in estuarine and marine environment. The species superficially resembles the human pathogen *Vibrio parahaemolyticus* and can be misidentified, if sole carbon source utilization studies are not performed (West and Colwell, 1984).

Occurrence and Distribution:

Singleton and Skermann (1973); Chumakova and Getelson (1975) reported the presence of luminous bacteria throughout the world; from tropical, temperate and polar regions and from the surface waters to depth of several thousand meters. In the Southern California three species of luminous bacteria were recorded in the nearshore surface waters of which two account for over 99% of the isolated strains. *Photobacterium fischeri* was found throughout the nearshore waters (1 - 5 Cells/ml) in winter whereas *Beneckea harveyi* was found predominant in summer (2.5 -

3.0 Cells/ml) indicating that there is a very high correlation between the surface temperature in the case of *B. harveyi* (Ruby and Nealson 1978). This author also mentions the presence of parasitic and enteric associations of *B. harveyi* . Jayabalan *et al.* (1978) reported the presence of symbiotic bioluminescent bacteria in different species of Leiognathids.

The occurrence of luminescent bacteria in *Penaeus indicus* from Cochin backwaters and southwest coast of India was reported by Krishnamoorthy (1979). Balakrishnan Nair *et al.* (1979) enlightened the presence of luminous bacteria by isolating and identifying them from Porto-Novo estuarine environment along the east coast of India. The presence of bioluminescent bacteria in the water column, sediments, shrimp and gastrointestinal tract of marine fishes from the semitropical estuarine environment of the east lagoon, Galveston island, particularly *B. harveyi* and the absence of *Photobacterium* was reported by O'Brien and Sizemore (1979). Shilo and Yetinson (1979) reported the presence of *B. harveyi* in coastal strip which is higher in nutrients and productivity than the open waters along the Mediterranean sea and the Gulf of Elat.

Luminous bacteria were isolated from North Atlantic Oceanic water samples taken from 1000m, which ranged in density from 0.4-30 CFU/100 ml (Ruby *et al.*, 1980). Jayabalan *et al.* (1982) reported the occurrence of luminescent bacteria in sediments of Vellar estuary along east coast of India. Characterisation of marine luminous bacteria isolated off the coast of China was done by Yang *et al.*(1983) A review of luminous microflora

associated with skin, gill and alimentary track of marine fishes was done by Venugopalan and Ramesh (1985). Chan *et al.* (1986) proved the presence of halophilic *Vibrios* in subtropical coastal waters of Hongkong and found nine strains of *V. harveyi* in these waters. Cao and Hu (1982) reported the distribution and composition of luminous bacteria in the estuary of the Chanjiang River.

Out of 463 strains isolated and identified upto the species level, *V. harveyi* (17.3%) was the predominant species of the genus *Vibrio* throughout sampling in the Seto inland sea of Japan (Venkateswaran *et al.*,1989). Makemson *et al.* (1992) Concluded the presence of luminous bacteria in seawater around the Islands of Bahrain dominated by *V. harveyi* and also reported that they have the capability to adhere to artificial fibrous surfaces. The luminous bacteria are useful biomarkers in the assessment of environmental health by its property of sensitiveness to toxicants (Ramaiah and Chandramohan, 1993).

Pathogenicity of luminescent bacteria in fin fishes:

Kraxberger *et al.* (1990) reported first time *V. harveyi* as an opportunistic fish pathogen which infects previously injured occular and dermal surface of common snook, *Centropomus undecimalis* that are held in captivity which also forms most common and numerically predominant form in snook cornea. Hispano *et al.* (1997) reported presence of *V. harveyi* on occular lesions of short sunfish (*Mola mola*).

Widespread mortalities in Atlantic spadefish and silver mullet were reported in Venezuela by *V. harveyi* with some external sign of disease, notably melanosis, abdominal distension and slow erratic swimming (Alvarez *et al.*, 1998). Fidopiastis *et al.* (1999) reported that fish pathogen *Vibrio Salmonicida* is closely related to the luminous bacteria *Vibrio fischeri* and *V. logei* and also suggested that *V. salmonicida* might also be capable of luminescence.

Pathogenicity of luminescent bacteria in shrimps:

O'Brien and Sizemore (1979) reported that the natural habitat of *V. harveyi* appears to be the gut of shrimps and marine sediments as to free-living environment. It has been categorised as an opportunistic pathogen causing disease (Lightner ,1983; Natividad and Lightner ,1992 ; Lightner *et al.*, 1992). Tansutapanit and Ruangpan (1987) reported mortality caused by *V.harveyi* in white shrimp larvae *P.merguiensis* in Thailand. Most Lumnious bacterial outbreak occurs mainly during rainy season in Indonesia (Sunaryanto and Marian,1986 ; Prayitno and Latchford,1995), indicating that environmental factors such as low temperature, salinity, pH and organic load might be involved in triggering disease outbreak. Also Farghaly (1950); Ramesh *et al.*, (1989) have shown that environmental factors influence the growth of luminous bacteria. Lavilla-pitogo *et al.* (1990) reported larval mortalities associated with luminescence in epizootic proportions in black tiger prawn *P. monodon* hatcheries in Panay Island, Philippines by *V. harveyi*

and *V. splendidus* and concluded by SEM observations that colonisation of these bacteria occurs especially on feeding apparatus and oral cavity of the larvae and suggested an oral route of entry for initial infection. The author also recorded when *V. harveyi* cells exceeded 10^2/ml in the water results in significant mortalities within 48 hrs.

The midgut content of spawners as well as pond reared juvenile contained numerous luminescent bacteria and they remain as a minor component of exoskeleton(Lavilla-Pitogo *et al.*, 1992). Otta *et al.* (1999) studied the bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. *Vibrio* spp. were the largest group in all ponds, in which *V. harveyi* constituted 5.2-36% of the flora. Wang *et al.* (1993); Alapide *et al.* (1997) isolated *V. harveyi* and *V. natriegens* from the hepatopancreas of Penaeus suffering from Red disease syndrome.

Shrimpscarrying both luminous (51%) and non-luminous (46%) *V. harveyi* in the hatcheries of Taiwan was studied and was correlated to the death of the shrimps with the appearance of luminous *V. harveyi.* (Song and Lee, 1993). Pillai and Jayabalan (1993), challenged *V. harveyi* with PL of *P. indicus* and proved that the pathogenicity depends on the strain and the susceptibility of the host. Even a dose of 10^5 CFU/ml for 96 hrs showed no clinical sign of luminescence, settling at bottom, opaque whiteness and weak swimming movement. The surface colonisation of luminescent *Vibrio* species is a common phenomenon in shrimp PL collected from Gulf region (Mohney *et al.*,

1994). Presence of *V. harveyi* infection in pond reared black tiger prawn in Thailand was revealed by Jiravanichpaisal *et al.* (1994).

Prayitno and Latchford (1995) reported that luminous bacteria are the major problem in shrimp hatcheries of Indonesia and other Asian countries. They also concluded that the virulence of these bacteria are related to age of the larvae, such that 25.28% for zoea, 47.08% for mysis and 51.50% for PL survival in 48 hrs exposure to pathogen , the *V. harveyi* exhibit a degree of host specificity. They also indicated that low salinity significantly increased the virulence of *V. harveyi* whereas the low pH exposure had the reverse effect. The presence of *V. harveyi* associated with the melanised fissures of cultured *P. indicus* was reported by Jawahar and Manley (1995). In hatcheries mass mortalities of *P. monodon* larvae was reported due to the presence of antibiotic resistant strain of *V. harveyi* along the east coast of India. It was also reported that mass mortality occurred during the month of June when ambient temperature ranged from 27-34°C. Moribund larvae showed large number of bacteria in the haemocoel (Karunasagar *et al.*,1994). Chen *et al.* 1995) concluded that when photobacteriosis appears in larval prawn, it leads to pathogenesis of zoea, mysis, PL and also infection of the juvenile and adult shrimps. It was supported with light and electron microscopic observations of infected individuals with degeneration and necrosis of cells, bacterial blocking of blood sinus and internal tissues, reductive phagocytosis and coagulation

function of lymphocytes. Isolation of *V. harveyi* from diseased kuruma prawn *P. japonicus* was reported by Liu *et al.* (1996 a) from Taiwan.

Biofilm:

Karunasagar *et al.* (1996) observed that *V. harveyi* can form biofilm on the three substrates they tested which include cement slab, high density polyethylene plastic and steel surface. They also reported that the bacteria in biofilm were found to be more resistant to chlorine disinfection when compared to their planktonic counterparts. Costerton *et al.* (1987) reported bacterial biofilm in nature and during disease.

Jawahar *et al.* (1997 b) studied the epibiotic infestation of luminous bacteria in hatchery reared mysis larvae of *P. indicus* and recorded no mortality despite a high level of 2.60 x 10^5/ml of luminous *V. harveyi* during infestation in the presence of ciproflaxacin. Severe mortalities due to luminescent *vibrios* occurred in pond cultured *P. monodon* juvenile particularly in the first 45 days of culture (Lavilla Pitogo *et al.*, 1998). Leano *et al.* (1998) did classification and characterisation of bacterial flora in the hepatopancreas of pond reared *P. monodon* juvenile affected with luminous bacteria and reported luminous bacterial load of the shrimp hepatopancreas with disease mean 2.4 x 10^1 CFU/hp and without mean 0.3 x 10^1 CFU/hp. In the tropical Australian crustacean, *Penaeus esculatus* isolation of *V.harveyi* was done and which on challenging with the infection of 200 ml with 10^6 cells/ml caused mortality in 12 hrs (Owen *et al.*, 1992).

Vandenbergha *et al.* (1998) studied the *Vibrios* associated with *P. chinensis* larvae in Chinese shrimp hatcheries and concluded the predominance of *V. alginolyticus* and *V. harveyi* from zoea stage onwards.The author also describes that the presence of *V. alginolyticus* might influence the pathogenicity of *V. harveyi* or might have an impact on the resistance of larvae to bacterial pathogen. Koch postulates was confirmed with *V. harveyi* infection in *P. vannamei* larvae (Robertson *et al.*, 1998). Bacteriological studies indicated the presence of more than 10^6 bact/ml in haemolymph of shrimp affected with white spot and these were predominantly *V. alginolyticus* and *V. harveyi* (Karunasagar *et al.*, 1998). Jawahar *et al.* (1999) using a new key reported the presence of 5 different species of luminous bacteria of Tutícorin (India) coastal and shrimp farm environments as *V. fischeri*, *V.harveyi*, *V. orientalis*, *V. splendidus* and *Photobacterium leiognathi* of which *V. harveyi* dominated constituting 88.66 - 90.93% of total luminous bacteria followed by *V. orientalis* (4.43 - 4.46%) and *V. splendidus* (2.11 - 3.35%).

CONTROL MEASURES:

The shrimp feeds were added with three forms of probiotics like fresh cell of *Bacillus S* II bacterium, fresh cells in normal saline solution and lyophilised form. Challenged study with *V. harveyi* after ten days of probiotic treatment showed 100% survival in treated group whereas control group had only 26% survival (Rengpipat *et al* ., 1998). Moriarty

(1998) proved that *Bacillus* species can control luminous *Vibrios* in penaeid aquaculture in Indonesian ponds in concentrations 1×10^4 - 1×10^5 / ml. Alabi *et al.* (1999) has recorded the efficacy of *V. harveyi* immersion vaccines as opposed to oral vaccination for *P. indicus* larvae. They also added the degree of protection offered increased with virulence of the pathogen from which the vaccine was made. Shrimp and Tilapia crop rotation was found effective in biological control of *V. harveyi* infection when compared to the use of other antibiotics and chemicals as the strains grow more resistant to these chemicals (Paclibare *et al.*, 1998). Filtration of resource water is highly recommended by many investigators for control of luminosis in shrimp hatchery.

Taxonomic key

Taxonomic key for identification of luminous bacteria was reported by different authors (Baumann *et al.*,1971;Reichelt and Baumann ,1973; Ruby and Nealson ,1978; West and Colwell ,1984; Alsina and Blanch, 1994). A simple key which provides faster identification of marine luminous bacteria based on 10 biochemical test was suggested and which enables to distinguish among the closely related marine luminous bacteria (Jawahar *et al.*, 1999).

Antibiotic sensitivity:

V. harveyi and *V. splendidus* isolated from diseased *Penaeus monodon* showed varied response to antibiotics. Of the 24 antibacterials tested only chloramphenicol, sodium nifurstyrenate and the nitrofurans (furazolidone, nitrofurazone, nitrofurantoin and prefuran) showed relatively low minimum inhibiting and bactericidal concentration (<25 µg/ml) also found that this bacteria showed varied response to chloramphenicol and prefuran and low sensitivity to OTC (Baticados, 1988; Baticados *et al.*, 1990).

The pattern of drug sensitivity of marine *Vibrio* showed multiple drug resistance than resistance to one or two drugs (Chandrika, 1983). A higher rate of resistance was found in Penicillin (22), Ampicillin (16), Erythromycin (16), Gentamycin (18), Septran (11) and Cephoron (19), (Chandrika and Ramachandran ,1992).

Sensitivity of luminous *V. harveyi* to nine selected antibiotics showed that the isolates were highly sensitive to gentamycin (10µg) and chloramphenicol (30 µg), moderately sensitive to OTC (30µg)and neomycin (30µg) and resistant to ampicillin (10µg), kannamycin (30µg), penicillin (10µg), streptomycin (10µg) and sulphadiazine (300 µg) (Pillai and Jayabalan, 1996). Jawahar *et al.* (1997) reported that chloramphenicol, nalidixic acid, OTC, trimethoprim and Ciproflaxacin were found effective against luminous *V. harveyi*.

Selective media used for enumeration of luminescent Bacteria:

Different types of media were used for the enumeration of the luminous bacteria. Modified Luminous agar (LA) was used for the enumeration of marine *Vibrios* by Reichelt and Baumann (1973).

The highly recommended and widely used media for luminous bacterial enumeration is Seawater Complex Agar (Ruby and Nealson, 1978). Luminous medium prepared with artificial seawater was reported by Baumann and Baumann (1981). Lavilla-Pitogo *et al.* (1990) recommended different media used for isolation of luminous bacteria. Nutrient Agar and Complex Seawater Agar were excellent for the recovery of luminescent bacteria from larval samples as well as for the display of luminescence. (Song and Lee, 1993; Jiravanichpaisal *et al.*, 1994; Leano *et al.*, 1998). Lavilla Pitogo *et al.* (1998) isolated *V. harveyi* from shrimp hepatopancreas using Thiosulfate Citrate Bile Salt Sucrose (TCBS) Agar supplemented with 2% NaCl. The same media was used with increased Nacl content (2.5%) by Mohney *et al.* (1994). Appropriate dilution were chosen for reading on Tryptic Soy Agar (TSA) Nutrient Agar (NA) (both with 1.5% NaCl) and Bromothymol Blue Teepol agar (BTB) incubated overnight and observed for luminescence during night (Lavilla Pitogo *et al.*,1990; Jawahar and Manley,1995). A selective medium for *V. harveyi* (*Vibrio harveyi* Agar) was suggested by Harris *et al.*(1996).

Bioluminescence

Some strains of *V. cholerae, V. harveyi, V. splendidus, V. fischeri, and V. logei* are able to emit light of blue green colour. The reaction leading to emission, catalysed by the enzyme luciferase has been shown similar in all prokaryotes (Nealson and Hastings,1979). In the presence of oxygen the substrate are reduced to Flavin Mono Nucleotide ($FMNH_2$), a long chain aldehyde (R.CHO: probably tetradecanal) which react.

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + H_2O + RCOOH + \text{Light}$$

Some luminous bacteria have the capacity to enter into a symbiotic association with marine animals. Of the five species only *V. fischeri* have been found in luminous organ of teleost fishes and squid (Nealson and Hasting,1979; Baumann and Baumann,1981; Hastings and Nealson,1981). Oxygen is an absolute requirement for luminescence, though only low level is required for the activity. Bacteria continue to emit light at a maximum rate even at low oxygen concentration in the medium of about 0.25% of air saturation (Harvey,1952).

Shadel and Baldwin (1992) reported that the expression of bioluminescence in the marine bacterium *V. fischeri* is controlled by a unique cell density dependent regulatory mechanism called autoinduction. The genes required for bioluminescence (the lux genes) are organised in

two divergently transcribed genes (Lux R - Lux ICDABEG) (lux CDABE) and the synthesis of diffusable signal molecule called autoinducer (lux I).

In summary, it can be stated that the knowledge of luminescent bacteria as an opportunistic pathogen in shrimp hatchery has accumulated slowly only from 1983 (Lightner) onwards eventhough other aspects of luminescence has been studied before in detail. Periodic reports of marine fish pathogens discovery interspersed with short term concentration on very special groups like luminous bacteria infections in shrimp larval stages mark the general trend of historical development and their ecology. The progression of critical studies since 1983 may be prognostic of the future achievements and developments to be expected.

3. MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 AREA OF STUDY:

The present study was conducted by monitoring the Vallarpadam hatchery samples (Resource water, larval rearing water and larvae of *P. monodon* and *P. indicus*) of Marine Product Export Development Authority (MPEDA) located along Cochin backwaters situated between $9^0$55'N to $9^0$60'N latitude and $76^0$15'E to $76^0$20'E (Fig. No.1) A full larval cycle of *P. monodon and P. indicus* were monitored. The source water for the hatchery is the Cochin backwaters (Plate No.1). Sea water of 29 ppt salinity after proper chlorination (15 ppm) is pumped into the larval rearing tank (Plate No.2). After every rearing operation the tanks are disinfected with 50-100 ppm of chlorine. The hatchery produces both *P. monodon* and *P. indicus* seeds depending on the availability of spawners.

P. monodon and *P. indicus* samples were also collected from landings of Cochin Fisheries Harbour (Fig. No.1).

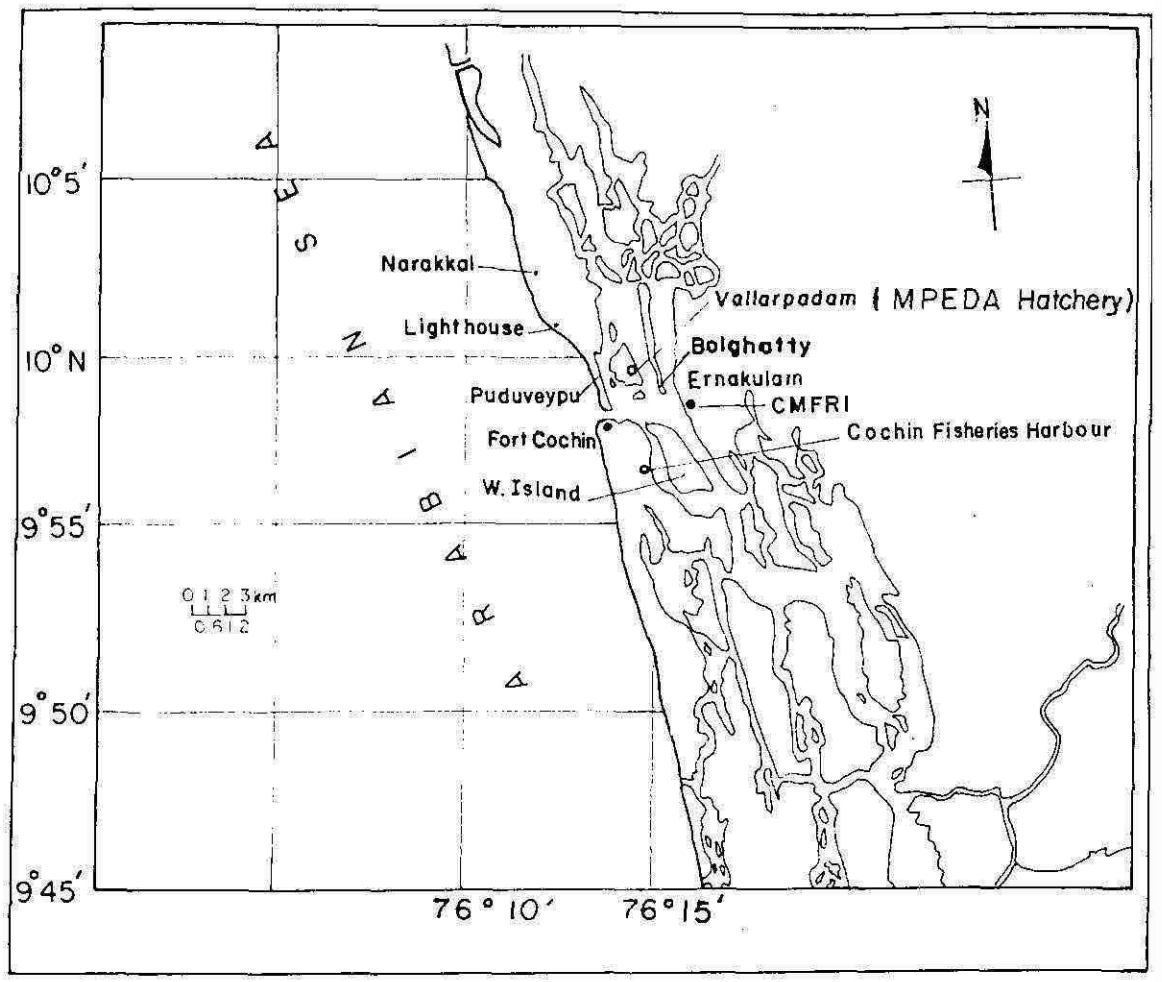


Fig. 1. Map showing the sampling stations

Plate-1: Resource \

Plate-2: Larval rearing tank (Vallarpadam hatchery)

TABLE 1 GENERAL PROCEDURES FOR SAMPLE SUBMISSION
LABORATORY TESTS FOR AQUATIC ANIMAL DISEASES

Diseases	Diagnostic methods	Samples
Bacterial haemorrhagic septicaemia	Bacterial identification	Live fish/shrimp
Vibriosis	Bacterial identification	Live fish/shrimp
Luminosis	Bacterial identification	Live shrimp larvae
Columnaris disease	Bacterial identification	Live fish
Bacterial gill disease	Bacterial identification	Live fish
Pseudotuberculosis	Bacterial identification	Live fish
Epizootic Ulcerative Syndrome (EUS)	Histopathology	Live fish
Protozoan parasite	Protozoa identification	Live fish/shrimp
Crustacean parasite	Crustacean identification	Live fish/shrimp
Yellow head disease	Histopathology	Live shrimp
White spot disease (SEMBV)	Histopathology Dot blot	Live shrimp
Monodon baculovirus (MBV)	Histopathology	Live shrimp
Hepatopancreatic virus infection (HPV)	Histopathology	Live shrimp
Infectious hypodermal and haematopoietic necrosis (IHHN)	Histopathology	Live shrimp

3.2 COLLECTION OF SAMPLES:

3.2.1 Collection of samples from Vallarpadam hatchery:

Samples for the present study were collected throughout the larval stages. The observation started from the zoea stage onwards and continued upto postlarvae. First cycle was of *P. monodon* and the second cycle was of *P. indicus*. Samples of larvae, larval rearing water (RW), nearshore water (NSW) were collected. RW and NSW were collected using sterile plastic bottle. Atleast ten live larval individuals were selected at random and were placed in sterile glass container which contained sterile seawater and were transported to the Bacteriology Laboratory of CMFRI for isolation of luminous bacteria within 1 hour.

3.2.2 Collection of wild shrimps from Cochin fisheries harbour :

Samples of *P. monodon* and *P. indicus* were also collected from Cochin Fisheries harbour. The shrimps were washed with sterile seawater and were collected asceptically in sterile plastic containers. The general procedures for sample submission is given in Table 1 for all aquatic animal diseases.

Care was taken to collect the samples following asceptic procedure. The samples were transported immediately to the bacteriology laboratory at CMFRI, Kochi for bacteriological investigation within 2 hours of sampling

3.3 BACTERIOLOGICAL INVESTIGATION:

Quantitative and qualitative analysis were carried out to know the occurrence, intensity and distribution of pathogenic luminescent bacteria. For isolation of luminescent bacteria, samples of the larvae were mascerated in the homogenising tube taking care of all asceptic procedures. Quantification of luminescent bacteria was also done from the larval rearing water and nearshore water. For isolation of luminescent bacteria from the gut of the wild shrimps, sterile scissors and forceps were used to cut open the abdomen and the gut was removed asceptically into sterile aged sea water.

3.3.1 QUANTITATIVE ANALYSIS:

Estimation of the luminous bacteria was done by serial dilution technique and pour plating technique (Rodina, 1972).

The water samples was filtered through 0.4 μ membrane filter. The filter paper was suspended in 99 ml of sterile aged seawater and shaken for 30 minutes at 150 r.p.m. in a bacteriological shaker (ORBITEK). After thorough shaking serial dilution were made according to standard procedures. Pour plating was done by using Seawater Complex Agar selective medium for luminescent bacteria (Ruby and Nealson ,1978), Nutrient agar medium (with 1.5% Nacl) (Lavilla-pitogo *et al.*,1990; Jawahar and Manley,1995). All ingredients used in this observation was from Hindustan-dehydrated media (HI-MEDIA-BOMBAY).

Seawater Complex Agar

Peptone 5 g; yeast extract 3g; glycerol 3 ml; DW. 250 ml; SW 750 ml; Agar 12 g.

Nutrient Agar with 1.5% NaCl

Peptone 10 g; beef extract 3 g; NaCl 5 g + 1.5% NaCl; Agar 20 g; DW 1000 ml. These ingredients were dissolved in distilled water by boiling and sterilised at 15 lbs for 30 min. The samples were pour plated and all plates were incubated overnight at room temp. (26-28°C) and colonies were enumerated by direct counts. Luminescence was observed in a dark room. Luminous colonies were marked on the outer surface of the lower dish of plates with glass markers. The plates were observed again after 48 hrs for additional luminous colonies. The marked colonies were subcultured using sterile toothpick and streaked on SWC agar slants.

After isolation, selected bacterial colony was subcultured in peptone water and kept as stock source. Young, 24 hr culture in the active logarithmic phase was used to carry out bio-chemical and physiological test leading to Identification based on the scheme of Alsina and Blanch (1994) and West and Colwell (1984).

BACTERIOLOGICAL PARAMETERS

3.4 MORPHOLOGICAL CHARACTERISATION

3.4.1 LUMINESCENCE: The plates were observed in dark for luminescence.

3.4.2 Gram's Staining:

Gram's Staining is a differential staining procedure which requires a stain and a counter stain. By this procedure, it is possible to divide the bacteria into Gram +ve, Gram -ve, Gram variable and Gram unreactive. A thin smear of the culture was prepared and air dried then it was heat fixed. The primary stain is crystal violet followed by addition of a mordant - iodine, which combines with the primary stain forming a crystal violet iodine complex. In the Gram Staining procedure, Gram -ve organism do not retain the primary dye. After decolourizing with 95% alcohol, Saffranin (counterstain) was added to the smear.

3.4.3 MOTILITY:

Motility was tested using Hanging-drop method.

3.5 BIOCHEMICAL TESTS:

Biochemical tests were done for identification and characterisation of the bacteria. Pure culture of bacteria was maintained (after isolation from Seawater Complex Agar and Nutrient Agar with 1.5% Nacl by observing in a dark room) in Peptone broth medium at RT. This pure culture helps in proceeding with different biochemical tests as follows.

3.5.1 0/129 SENSITIVITY:

0/129 is a pteridine compound (2, 4 diamine diisopropyl pteridine and is vibriostatic. The culture was spreadplated by evenly inoculating the

surface of TSA-medium and the disc was placed (which contained 150µg/disc of 0/129 compound) over the culture and incubated at RT for 24 hrs. until growth is obtained. If +ve clear inhibition zone will be formed around the disc.

3.5.2 OXIDASE TEST:

Oxidase test done to detect the presence of certain respiratory enzyme like cytochrome oxidase in the bacteria that will catalyse the transport of electrons between the electron donors in the bacteria and a redox dye. This dye is reduced to a deep purple colour.

For conducting the test, a filter paper strip in which a little reagent (freshly prepared 1% N^1, N^1, N^1, N^1 - tetramethyl paraphenylene diamine dihydrochloride) was poured. A loop of culture is taken and streaked into this. Immediate appearance of a deep purple colour indicated positive reaction for the presence of oxidase enzyme.

3.5.3 NITRATE REDUCTION TEST:

The ability to reduce nitrate, was tested in an ordinary peptone broth containing 0.3% potassium nitrate inoculated with the culture. Turbidity was checked after the incubation period and the nitrate reduction was tested with 0.1 ml of nitrate reagent (Alpha naphthylamine, reagent A; Sulphanilic acid, reagent B). Immediately before use, mix equal volumes of solutions A and B to give the test reagent.

3.5.4 'Voges-Proskauer' test:

"MR-VP" broth (Himedia) was inoculated with the test culture and incubated for 5-7 days at room temperature and 'Voges Proskauer' reactions were carried out.

0.6 ml of 5% solution of alpha-naphthol in ethanol and 0.2 ml of 40% KOH was added to 1 ml of `MR-VP' cultured broth. Pink colour in 2-5 ml and deepening to magenta or crimson in 30 minutes indicated VP +ve and -ve if it remains colourless.

3.5.5 "OXFERM"; O/F TEST; HUGH & LEIFSON'S TEST:

Method to distinguish aerobic and anaerobic breakdown of carbohydrates. This test was carried out in a media known as H&L medium, whose composition as follows, peptone -1 % Sodium chloride 0.5%, Glucose 1%, Agar-agar 0.3%, Distilled water 100 ml, Phenol red of 0.1% solution, K_2HPO_4 -0.3%.

This method (Hugh & Leifson, 1953) depends upon the use of semi-solid tubed medium containing the carbohydrate together with a pH-indicator. If acid is produced only at the surface of the medium, where conditions are aerobic, the attack on the sugar is oxidative.

If acid is found throughout the tube, including the lower layers where conditions are anaerobic the breakdown is fermentative.

Method: Duplicate tubes of solidified medium are inoculated by stabbing, one tube was promptly covered with a layer of sterile liquid paraffin to make it anaerobic.

This medium can also be used for detection of gas production and motility.

3.5.6 H_2S PRODUCTION TEST:

Some organisms decompose sulphur-containing aminoacids to form H_2S among the products. L-cystine, the sulphur containing amino acid was prepared in liquid broth along with peptone, sodium chloride and beef extract for detection of H_2S production. The test organisms were inoculated in the liquid medium and lead acetate paper impregnated with saturated lead acetate solution was kept at the top of the tube. The culture was incubated for 24 hrs at room temperature and blackening of the lead acetate paper was noted.

3.5.7 GELATIN LIQUEFICATION:

The gelatin medium (peptone - 1%, Beef extract - 0.2%; Gelatin - 12%; Seawater - 100 ml) was sterilised by Tyndallisation . The medium was inoculated heavily with the test organisms by stab-inoculation and incubated for 96 hrs.

After the incubation period the liquefication of gelatin was tested by keeping the test cultures in refrigerated condition (4°C) for 30 min along with control before reading the results.

3.5.8 CATALASE TEST:

10-20% Hydrogen peroxide (H_2O_2) was taken in a test tube, and a loop containing bacterial culture was dipped into it. The catalase +ve reaction showed a stream of oxygen bubbles coming out of the loop, whereas the -ve reaction fails to form the oxygen release.

3.5.9 INDOLE PRODUCTION TEST:

The tryptone broth medium was inoculated with one loopful of 24 hrs nutrient broth culture and incubated for 72 hrs. 48 hrs are ordinarily sufficient if good growth is obtained. To test the culture for indole production 5cc of Kovac's reagent was added. The appearance of a deep red ring in the reagent layer indicated the presence of indole.

3.5.10 AMINOACID DECARBOXYLASE TEST:

This test is based on the ability of some bacteria to decarboxylate an aminoacid to the corresponding amine with the liberation of CO_2. The Amino acid decarboxylase basal medium (Peptone 5 g; Meat extract 5 g; Glucose 0.5 g; Pyridoxal 5 mg; Bromocresol purple (1 in 500 soln) 5ml; Cresol red (1 in 500 soln) 2.5 ml; Distilled water 1000 ml) was prepared. Divided this basal medium aminoacids L-lysine hydrochloride, L-ornithine hydrochloride, L-arginine hydrochloride were added. Then this medium was inoculated and sterile liquid paraffin was added to provide a thin layer. The cultures were incubated and read daily for four days. +ve tubes becomes violet and -ve tubes remained yellow.

3.5.11 SUGAR FERMENTAION

The peptone water (Andrade's peptone water, Himedia) to which 1% of the respective sugar was added, was sterilised at 10 lbs/min. After sterilisation the medium was inoculated and incubated aerobically for 24 hrs. The colour change indicated sugar fermentation and gas formation in the Durham's tube was also noted. The different sugars used in this experiments were Glucose, Lactose, Meso-Inositol, Sucrose, Arabinose and Cellobiose.

3.5.12 SODIUM CHLORIDE - TOLERANCE TEST:

Tolerance to NaCl (0%, 3.5% and 10%):

0%, 3.5% and 10% NaCl in peptone broth (Peptone - 1%) was inoculated with test culture incubated for 24 hrs at RT. The turbidity indicated the growth in that medium.

3.5.13 GROWTH IN TCBS:

The test cultures were streaked on to Thiosulphate Citrate Bile salt Sucrose Agar (TCBS) and growth was observed after 24 hour incubation at RT.

3.5.14 HAEMOLYSIS TEST:

This test was done to find out haemolytic activity of the isolate. This experiment was done with goat blood, taking the basal medium either Blood-Base Agar or Tryptone Soya Agar.

3.5.14.1 Collection of Blood:

The goat blood was collected in a sterilised conical flask containing Alsever's solution from the slaughter house.

Composition of Alsever's Solution.

NaCl - 2.1 gm, Tri-Sodium Citrate - 4 gm, Citric Acid - 0.275 gm, Glucose/Dextrose - 10.25 gm, D.W - 500 ml.

500 ml Alsever's Solution for 200 ml of blood.

3.5.14.2 Media Preparation & Plating:

The freshly collected blood was added to sterile medium (TSA), when it was at 45 - 50^{o} C at the rate of 7% v/v.

Pour plating or streaking of the pure bacterial culture was carried out in the sterilised petriplates. The plates were observed after 24 hr to find out the type of haemolysis.

Alpha haemolysis (α): An indistinct zone of partial destruction of red blood cells around the colony.

Beta haemolysis (β): A clear colourless zone around the LB colonies in which blood cells have undergone complete dissolution.

Gamma haemolysis (γ): No apparent haemolytic activity or discolourartion produced around the colony.

3.6 ANTIBIOTIC SENSITIVITY TEST:

Antibiotic sensitivity of luminescent *Vibrios* was tested with young culture (18 hrs) to find out the resistance pattern of luminescent *Vibrios* to various antibiotics. The test cultures were swabbed on the Muller-Hinton agar and the discs (Hi - Media) were placed on the surface of the medium and the plates were incubated. The antibiotic discs were of different potency as follows:

Gentamycin (10 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Neomycin (30 µg), Ampicillin (10µg), Penicillin (10µg), Streptomycin (10µg),Nalidixic acid (30µg),Ciproflaxacin(10µg),and Co-trimoxazole(25µg)

The antibiotics diffuses through the agar occupying a circular zone around the original spot. The bacteria grow on the agar surface in all places except in circular zone where the antibiotics is present. Size of the zone is related to concentration of the antibiotics. The size of the 'halo' was noted and mentioned as sensitive (S) or Resistant (R) after comparing with zone size interpretative chart.

3.7 PATHOGENICITY STUDIES:

Advanced *P. indicus* postlarvae (15-20 mm) and postlarvae of *P. monodon* (10-15 mm) were collected from uninfected healthy stock from the MPEDA-Vallarpadam hatchery. Subsequently they were acclimatised to the laboratory conditions in glass aquaria for 5 days with the salinity of 30 ppt. The larvae were kept under continuous aeration

and were fed adequately with shrimp feed. The excess feed and faecal matter were siphoned out daily.

3.7.1 EXPERIMENTAL SET UP:

Batches of 5 healthy larvae were introduced into glass trough containing 2 litres of chlorinated, membrane filtered, sterilised sea water with the same salinity as used for acclimatization. The experiments were carried out in replicates with control (plate No. 6). Continuous aeration and proper feeding was done during the experiment.

3.7.2 Preparation of appropriate dilution of luminous bacteria suspension and administration pattern:

Strains of luminescent *V. harveyi* and *V. splendidus* I isolated in the present study (Strain No. 1 and 5) were sub-cultured and grown in 100 ml of Seawater Complex Broth. These strains in their logarithmic phase (Plate 4) were centrifuged at 5700 r.p.m. for 7 min. The bacterial pellet was rinsed in sterile seawater and diluted to give final concentration of 10^3, 10^4 and 10^5 Cells/ml and were added to the glass trough. The troughs were checked once in every 12 hours to record mortality, if any until the termination of the experiment after 48 hours. (Pillai and Jayabalan, 1996)

3.7.3 Re-isolation of Luminous bacteria from the experimental larvae:

After the experimental period, selective isolation of the luminous *vibrios* was done in SWC agar by pour plating from larvae in the control trough as well as in the experimental set up.

4. RESULTS

RESULTS

4.1 QUANTIFICATION OF LUMINESCENT BACTERIA

4.1.1 Monitoring luminescent bacteria in Vallarpadam (MPEDA) hatchery:

4.1.1.1 *P. monodon* larval cycle:

First observation of *P. monodon* larval cycle was made and nearshore water for 'LB' on 14th of March 2000 (4th day of the larval cycle). Only nearshore water harboured luminescent bacteria ('LB') in Nutrient agar, the count being 5×10^4 CFU/ml, out of 15×10^4 CFU/ml non luminescent bacteria ('NLB') recorded, which showed the occurrence of 'LB' as 33.33% of the total. All the other samples like rearing water of zoea and zoea sample were found absent for 'LB' eventhough the 'NLB' count ranged from 26×10^4 CFU/ml and 212×10^4 CFU/g in SWC Agar respectively (Table No. 2).

In subsequent larval stages like mysis and post larvae samples of *P. monodon* 'LB' was not encountered both in SWC agar and Nutrient agar eventhough other heterotrophs were found predominant in 10^3, 10^4 and 10^5 dilutions.

TABLE. 2 QUANTIFICATION OF LUMINESCENT BACTERIA IN VALLARPADAM HATCHERY- PENAEUS MONODON LARVAL CYCLE

		SWCA			NA		
	SAMPLE	**10^3**	**10^4**	**10^6**	**10^3**	**10^4**	**10^6**
ZOEA	NSW	TNTC	102	24	120	15 5	NIL
	RWZ	TNTC	26	2	TNTC	16	NIL
	ZOEA	TNTC	212	114	140	109	12
MYSIS	NSW	40	36	16	5	2	NIL
	RWM	93	22	10	22	6	2
	MYSIS	TNTC	TNTC	28	TNTC	TNTC	4
PL	NSW	160	31	25	112	20	9
	RWPL	TNTC	39	8	TNTC	18	2
	PL	TNTC	TNTC	80	TNTC	49	10

SWCA-SEAWATER COMPLEX AGAR
NA – NUTRIENT AGAR
NSW- NEARSHORE WATER
RWZ-REARING WATER ZOEA
RWM-REARING WATER MYSIS
RWPL-REARING WATER POST LARVAE
PL-POST LARVAE
TNTC- TOO NUMEROUS TO COUNT

- OCCURRENCE OF "LB"

TABLE.3 QUANTIFICATION OF LUMINESCENT BACTERIA IN VALLARPADAM HATCHERY- PENAEUS INDICUS LARVAL CYCLE

SEAWATER COMPLEX AGAR

	SAMPLE	10^3		10^4		10^5	
		DIR	FILT	DIR	FILT	DIR	FILT
ZOEA	NSW	TNTC	39	2	15	2	2
	RWZ	17	20	6	11	2	5
	ZOEA	10		6		3	
MYSIS	NSW	26	47	9	38	4	3
	RWM	21	38	11	30	6	12
	MYSIS	8		3		2	
PL	NSW	TNTC	TNTC	42	112	19	52
	RWPL	TNTC	48	11	21	3	NIL
	PL	13		6		NIL	

NUTRIENT AGAR

	SAMPLE	10^4		10^5		10^6	
		DIR	FILT	DIR	FILT	DIR	FILT
ZOEA	NSW	TNTC	25	2	4	NIL	1
	RWZ	9	TNTC	1	2	2	1
	ZOEA	29		12		11	
MYSIS	NSW	TNTC	80	12	30	2	17
	RWM	32	TNTC	22	NIL	5	NIL
	MYSIS	39		9		NIL	
PL	NSW	TNTC	TNTC	32	77	12	18
	RWPL	34	50	19	22	4	7
	PL	24		11		3	

DIR- Direct FILT-Membrane Filtered

TABLE 4 QUANTIFICATION OF LUMINESCENT BACTERIA IN WILD SHRIMPS

SPECIES	10^2	%	10^3	%	10^4	%	10^5	%	10^6	%	10^7	%	10^8	%
P.MONODON 12-04-00	360	8.3	240	3.3	39	7.7	15		2	0	Nil		Nil	
14-04-00	76	21	35	11.4	14		Nil		Nil		Nil		Nil	
15-04-00	89	5.6	51	15.7	9		Nil		Nil		Nil		Nil	
P.INDICUS 12-04-00	TNTC		72	13.8	31	9.6	3		Nil		Nil		Nil	
14-04-00	62		21		Nil		Nil		Nil		Nil		Nil	
16-04-00	57	3.5	38	10.5	5		Nil		Nil		Nil		Nil	

- OCCURRENCE OF "LB"

TABLE.5 BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES

CHARACTERISTICS	1	2	3	4	5	6	7	8	9	10	11	12	13
GRAM REACTION	-	-	-	-	-	-	-	-	-	-	-	-	-
LUMINESCENCE	+	+	+	+	+	+	+	+	+	+	+	++	+
MOTILITY	+	+	+	+	+	+	+	+	+	+	+	+	+
SENSITIVITY O/129(μ G)	S	S	S	S	S	S	S	S	S	S	S	S	S
CYTOCHROME OXIDASE	+	+	+	+	+	+	+	+	+	+	+	+	+
NITRATE REDUCTION	+	+	+	+	+	+	+	+	+	+	+	+	+
VOGES-PROSKAUER	-	-	-	-	-	-	-	-	-	-	-	+	-
FERMENTATION OF GLUCOSE	+	+	+	+	+	+	+	+	+	+	+	-	+
H_2S PRODUCTION	+	+	+	+	+	+	+	+	+	+	+	-	+
GELATIN LIQUEFICATION	+	+	+	+	+	+	+	+	+	+	+	+	+
CATALASE	+	+	+	+	+	+	+	+	+	+	+	+	+
INDOLE	+	+	+	+	+	+	+	+	+	+	+	-	+
AMINO ACID: ARGININE DIHYDROLASE	-	-	-	-	-	-	-	-	-	-	-	-	-
LYSINE DECARBOXYLASE	+	+	-	+	-	-	+	+	+	+	+	-	-
ORNITHINE DECARBOXYLASE	+	+	-	+	-	-	-	-	-	+	+	-	-
SUGARS: GLUCOSE	+	+	+	+	+	+	+	+	+	+	+	+	+
SUCROSE	+	+	+	+	+	+	+	+	+	+	+	+	+
ARABINOSE	-	-	-	-	-	-	-	-	-	-	-	-	-
INOSITOL	-	-	-	-	-	-	-	-	-	-	-	-	-
CELLOBIOSE	+	+	+	+	+	+	+	+	+	+	+	+	+
GROWTH I N 0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-
3.5%NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
10%NaCl	-	-	-	-	-	-	-	-	-	-	-	-	+
GROWTH IN TCBS	Y	G	G	Y	Y	G	G	G	G	G	G	G	Y
β - HAEMOLYSIS	+	+	+	+	+	-	-	-	-	+	-	+	+

+ POSITIVE - NEGATIVE ++ HIGHLY POSITIVE

4.1.1.2 *P. indicus* larval cycle:

Table No. 3 illustrates the data of 'LB' of *P. indicus* larval cycle sampled on 3rd of April 2000 (4th day of the larval cycle). 'LB' was found absent in NSW, LRW and stages like zoea, mysis and PL.

4.1.2 Quantitative analysis of Luminescent bacteria in wild shrimps:

4.1.2.1 *P. monodon*:

The count of nonluminescent bacteria ranged from 360 x 10^2 CFU/gm - 2 x 10^6 CFU/gm in wild shrimp gut (Table no. 4) whereas luminescent strains ranged from 30 x 10^2 CFU/gm - 3 x 10^4 CFU/gm in SWC agar. The paucity of 'NLB' and 'LB' in 10^5, 10^6, 10^7 and 10^8 may be due to the selective ingredient glycerol (0.3%) incorporated in the medium.

In the second observation the count of 'NLB' varied from 76 x 10^2 CFU/gm -14 x 10^4 CFU/gm whereas 'LB' ranged from 16 x 10^2 CFU/gm- 4 x 10^3 CFU/gm. In the third observation 'NLB' ranged from 89 x 10^2 CFU/gm -9x10^4 CFU/gm whereas 'LB' ranged from 5 x 10^2 CFU/gm- 8 x 10^3 CFU/gm.

In the present observation the percentage composition of 'LB' in the gut of *P. monodon* was maximum of 21% in 10^2, 15.7% in 10^3 and 7.7% in 10^4 dilutions.

Plate-3:Occurrence of Luminescent bacteria in SWC Agar

Plate –4: Luminescent bacteria in logarithmic phase in SWC broth

A) In Day light **B) In Darkness**

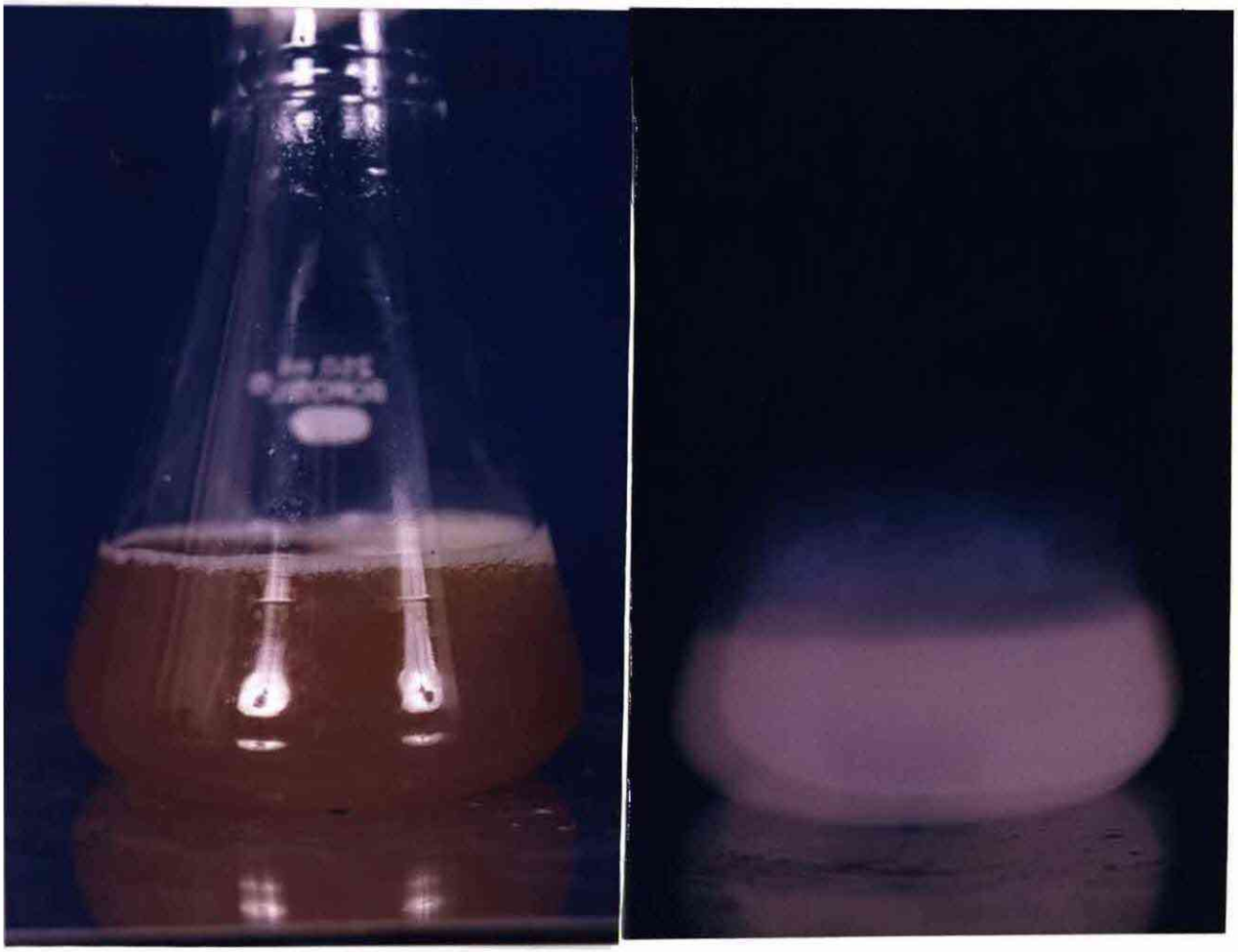


Plate-5: Drug Sensitivity of Luminescent bacteria

A: In Day light

B: In Darkness

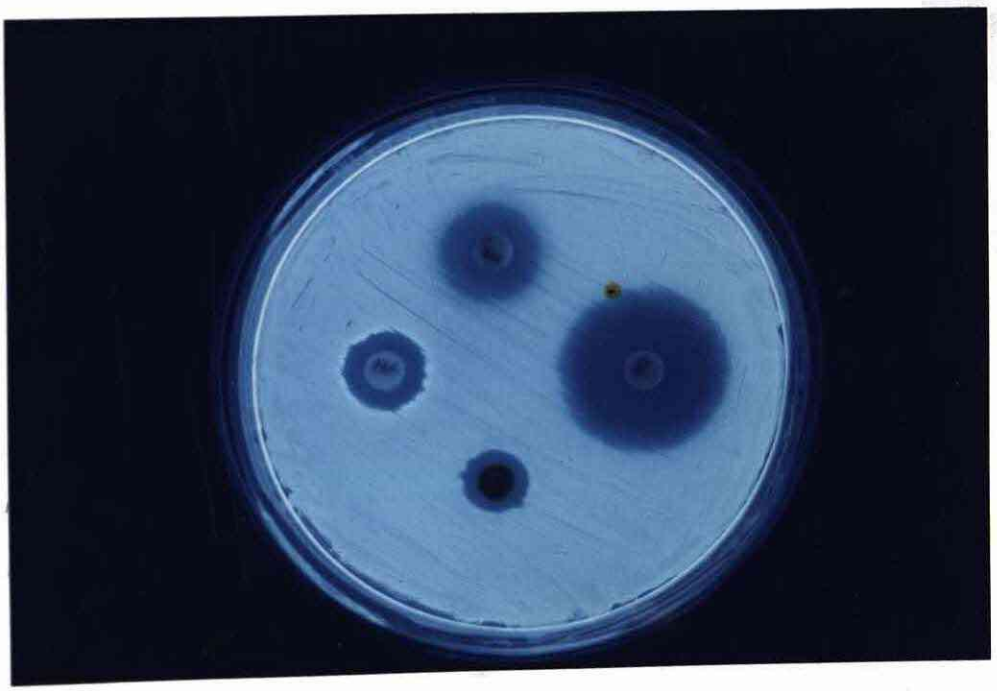


Plate –6: Pathogenicity study- Experimental set up

Plate-7: Dead P.indicus larvae after immersion experiment(10^5 dilution after 48hrs)


I
II
C
III

4.1.2.2 *P. indicus*

The count of 'NLB' in the wild shrimps gut ranged from 72 x 10^3 CFU/gm - 3 x 10^5 CFU/gm whereas 'LB' count was ranging from 10 x 10^3 CFU/gm- 3 x 10^4 CFU/gm(Tabe No.4)

In the second observation, it was not possible to retrieve 'LB' eventhough the 'NLB' count ranged 62 x 10^2 CFU/gm-21x10^3 CFU/gm. In the third observation the 'NLB' ranged from 57 x 10^2 CFU/gm-5 x 10^4 CFU/gm whereas the 'LB' ranged from 2 x 10^2 CFU/gm- 4 x 10^3 CFU/gm.

The percentage composition of 'LB' in the gut of *P. indicus* was maximum of 3.5% in 10^2, 13.8% in 10^3 and 9.6% in 10^4 dilutions.

4.2 MORPHOLOGICAL CHARACTERS:

The size of colony in Seawater complex agar ranged from 2-3 mm in diameter after overnight incubation at RT. They were cream-coloured, shiny, smooth, circular and raised colonies. The colonies were brightly luminescent giving bluish green during darkness. (plate No.3). The bio-luminescent activity started by 4 hours and gradually increased. The degree and time duration of luminescence was peak at 18 hours and then it reduced gradually but remained for 72 hrs. (Plate No.4) Bioluminescence varied with different isolated luminescent strains.*Photobacterium leiognathi* gave maximum bioluminescence in the present study.

The cells were Gram-negative, motile, short rods.

4.3 BIOCHEMICAL CHARACTERISTICS:

All the strains were oxidiase positive and sensitive to 0/129 compound (150 µg) and were actively reducing nitrate to nitrite and ammonia. 92.3% of the isolated strains produced indole and were voges-praskauer positive. 92.3% of the strains fermented glucose and produced H_2S. 100% of the strains hydrolysed gelatin and produced catalase.(Table No.5)

4.3.1 Aminoacid decarboxylation by 'LB':

All the isolates were not harbouring the enzyme dihydrolase to hydrolyse arginine whereas 61.53% isolates were having the hydrolysing potential of the aminoacid lysine. 38.5% were capable of decarboxylating ornithine. Based on the aminoacid decarboxylase the strains were classified into *V. harveyi* 38.46%, *V. splendidus* I 30.76%, *V. orientalis* 23.03%, *Photobacterium leiognathi* 7.69%(Table No.6).

4.3.2 Sugar-fermentation by 'LB':

Out of the six sugars tested for saccharolytic activity glucose, sucrose, cellobiose were fermented by all the 13 cultures whereas lactose, arabinose, inositol were not fermented by any of the 'LB' isolated.

4.3.3 Sodium-chloride Tolerance by 'LB':

NaCl tolerance was tested with 0%, 3.5% and 10%, in which 3.5% NaCl concentration was found suitable for all the strains as 100% growth was obtained in all the cultures. In 0% NaCl concentration no growth was observed and in 10% NaCl concentration only one culture showed growth in trace amount.

4.3.4 Growth in TCBS:

69.2% of the strains showed bluish green colour growth in TCBS and 30.76% of the strains showed yellow colour growth in TCBS and one strain showed very poor growth ,which was designated as *Photobacterium*.

4.4 ANTIBIOTIC SENSITIVITY TEST OF 'LB'

TableNo. 7 illustrates the results, obtained by testing the antibiotic sensitivity of the isolated 13 cultures. The antibiotic sensitivity study indicated the antagonistic activity of the strains towards the various antibiotics tested. All the isolated cultures showed 100% sensitivity towards the antibiotics chloramphenicol (30µg), and streptomycin (10 µg). 84.7% of the strains were sensitive to nalidixic acid (30 µg) and co-trimoxazole (25 µg). 30.76% of strains were sensitive towards Tetracycline, (30 µg), gentamycin (10µg) and 100% resistance was recorded for ampicillin (10µg), penicillin (10µg), ciproflaxacin (10µg)(Plate No.5). Multiple drug resistance was prevalent among the strains.(Table No.8)

TABLE.6 PERCENTAGE COMPOSITION OF LUMINESCENT BACTERIA

SPECIES	PERCENTAGE
VIBRIO HARVEYI	38.46
VIBRIO SPLENDIDUS I	30.76
VIBRIO ORIENTALIS	23.07
PHOTOBACTERIUM LEIOGNATHI	7.69

TABLE 7 ANTIBIOTIC SENSITIVITY OF LUMINESCENT STRAINS

DRUGS	1	2	3	4	5	6	7	8	9	10	11	12	13
CHOLARMPHENICAL (30µG)	S	S	S	I	S	S	S	S	S	S	S	S	S
STREPTOMYCIN (10µG)	I	I	I	I	S	I	S	S	S	S	S	S	S
TETRACYCLINE (30µG)	R	R	R	I	R	I	R	I	I	R	R	R	R
AMPICILLIN (10µG)	R	R	R	R	R	R	R	R	R	R	R	R	R
PENICILLIN (10µG)	R	R	R	R	R	R	R	R	R	R	R	R	R
NALIDIXIC ACID (30 µG)	S	S	R	I	S	S	I	S	I	S	S	I	R
CIPROFLAXACIN (10 µG)	R	R	R	R	R	R	R	R	R	R	R	R	R
GENTAMYCIN (10 µG)	R	I	R	R	R	R	S	S	R	R	R	R	S
CO-TRIMOXAZOLE (25 µG)	S	S	S	I	S	I	S	S	I	S	R	R	I

S - SENSITIVE R - RESISTANT I - INTERMEDIATE

TABLE 8 DRUG RESISTANCE PATTERN OF 'LB'

CULTURE	RESISTANCE	INTERMEDIATE	SENSITIVE
1	TE,AM,PG,CX,G	SM	CH,NA,BA
2	TE,AM,PG,CX,G	SM	CH,NA,BA
3	TE,AM,PG,NA,CX,G	SM	CH,BA
4	AM,PG,CX,G	CH,SM,TE,NA	BA
5	TE,AM,PG,CX,G		CH,SM,NA,BA
6	AM,PG,CX,G	SM,TE,BA	CH,NA
7	TE,AM,PG,CX	NA	CH,SM,G,BA
8	AM,PG,CX	TE	CH,SM,NA,G,BA
9	AM,PG,CX,G	TE,NA,BA	CH,SM
10	TE,AM,PG,CX,G		CH,SM,NA,BA
11	TE,AM,PG,CX,G,BA		CH,SM,NA
12	TE,AM,PG,CX,G,BA	NA	CH,SM
13	TE,AM,PG,NA,CX	BA	CH,SM,G

CH-CHOLARMPHENICAL
SM-STREPTOMYCIN
TE-TETRACYCLINE
AM-AMPICILLIN
PG-PENCILLIN
NA-NALIDIXIC ACID
CX-CIPROFLAXACIN
G-GENTAMYCIN
BA-CO- TRIMOXAZOLE

TABLE.9 PATHOGENICITY STUDIES

SPECIES TREATED	INOCULUM LEVEL (CELLS/ML)	% SURVIVAL AFTER TERMINATION (48 hrs)	LUMINESCENCE (OBSERVED IN DARK)		RE-ISOLATION OF 'LB' FROM LARVAE
			WATER	LARVAE	
V.HARVEYI					
P.INDICUS (15-20mm)	0(control)	100%	Nil	Nil	Nil
	10^3	100%	Nil	Nil	+
	10^4	100%	+	Nil	+
	10^5	40%	+	+	+
P.MONODON (10-15 mm)	0(control)	100%	Nil	Nil	Nil
	10^3	100%	Nil	Nil	+
	10^4	100%	+	Nil	+
	10^5	20%	+	+	+
V. SPLENDIDUS I					
P.INDICUS (15-20mm)	0(control)	100%	Nil	Nil	Nil
	10^3	100%	Nil	Nil	+
	10^4	80%	+	Nil	+
	10^5	40%	+	+	+
P.MONODON (10-15 mm)	0(control)	100%	Nil	Nil	Nil
	10^3	100%	Nil	Nil	+
	10^4	100%	+	Nil	+
	10^5	40%	+	+	+

Fig. 2 **Percentage mortality of Penaeid post - larvae challenged against *Vibrio harveyi***

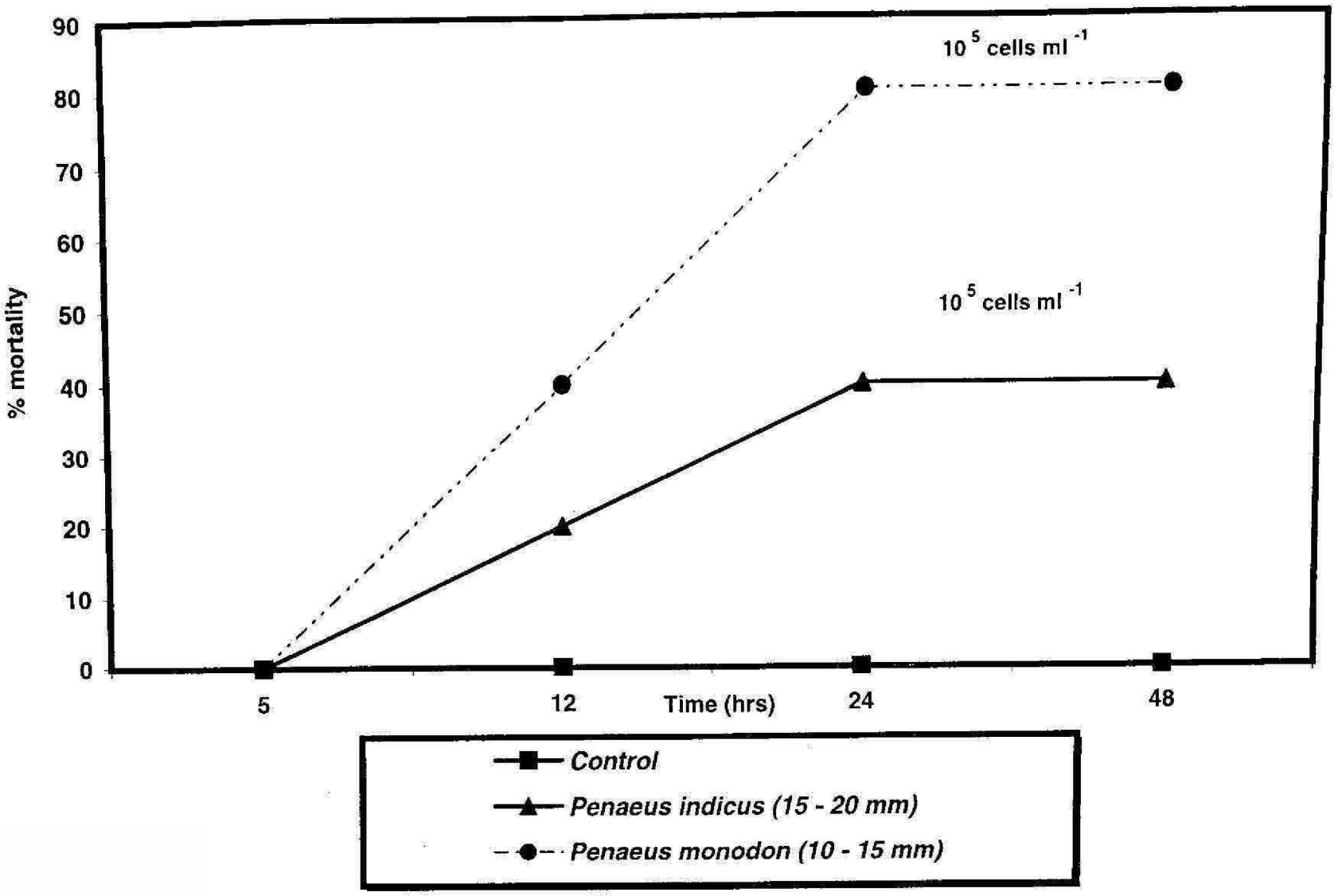
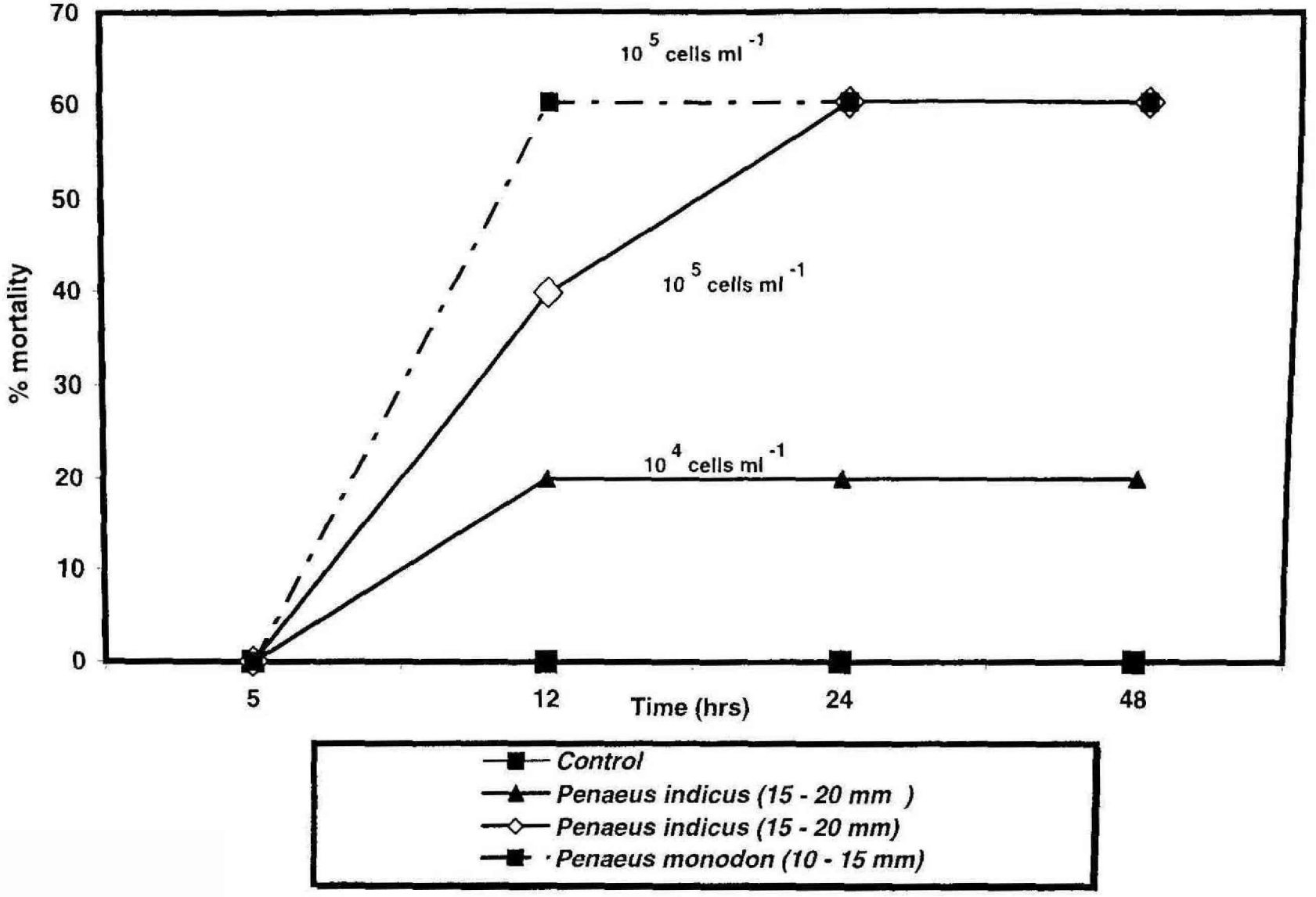


Fig . 3. Percentage mortality of Penaeid Post larvae challenged against *Vibrio splendidus* 1



4.5 PATHOGENICITY STUDIES:

4.5.1 *V. harveyi:*

P. indicus (15-20 mm) inoculated with *V. harveyi* in 10^3, 10^4 and 10^5 cells/ml, 60% mortality was obtained in of 10^5 cells/ml inoculum (Plate No. 7) and luminescence was observed only in water in 10^4 cells/ml concentration and in both water and larvae in 10^5 cell/ml concentration.

Luminous bacteria were re-isolated from homogenized larvae obtained from the test doses, even from those which apparently were not luminescent and which remained active until the termination of the experiment. Larvae from the control showed no sign of luminescence (Table No.9).(Fig.No. 2)

In the case of *P. monodon* (10-15 mm) with *V. harveyi* upto 80% mortality was obtained at 10^5 cells/ml inoculum. Luminescence was also observed in water and larvae. It was possible to re-isolate the luminous bacteria from the moribund and dead larvae.

4.5.2 *V. splendidus* I :

In *P. indicus* (15-20 mm) 20% mortality was recorded at 10^4 cells/ml and 60% mortality at 10^5 cells/ml. Luminescence was observed in water at 10^4 cells/ml concentration and both larvae and water in 10^5 cells/ml concentration. It was possible to retrieve luminescent bacteria from the moribund and dead larvae (Table No.9)(Fig.No. 3)

5. DISCUSSION

DISCUSSION

The principle interest of this investigation was to study luminescent bacteria as there is no data from shrimp hatcheries of Cochin area eventhough some reports are there from the wild *P. indicus* of Cochin Backwaters (Krishnamoorthy, 1979) and estuarine environments of Porto-Novo (Balakrishnan Nair *et al.*, 1979; Jayabalan *et al.*, 1982). Jawahar Abraham *et al.*(1999) studied the percentage composition of 'LB' in coastal and shrimp farms of Tuticorin coast. Pillai and Jayabalan (1993) isolated *V. harveyi* from *P. indicus* from Mangalore fish landings.

Occurrence and distribution of 'LB' was observed in Vallarpadam shrimp hatchery during March 2000 in different larval stages of the life cycle of *P. monodon* and *P. indicus*, and also rearing water and resource water, monitoring of 'LB' was also done in wild shrimp. Totally 13 'LB' cultures were isolated and their biochemical and physiological characters have been screened. Also antibiotic sensitivity and β - haemolytic activity was studied to know their virulence. Strains of *V. harveyi* and *V. splendidus* I were selected based on the above two characteristics for their pathogenicity studies in *P. monodon* and *P. indicus* postlarvae.

So far 'LB' of Cochin area were unexplored for their pathogenicity. Many of the 'LB' considered to be responsible for

diseases are normal flora of the environment and are regarded as opportunistic pathogens. Only *V. harveyi* has been studied often for their pathogenicity. Though all 'LB's are not responsible for major losses, 3 species are associated with pathogenicity of cultured larvae and adult shrimps. Although the etiological agent of pathogenicity was reported as *V. harveyi* and *V. splendidus* I the exact epizootiology and pathology of these diseases remain unknown. Many of these isolates may occasionally be involved in infections as a result of excess stress caused by other environmental factors. The pathogenicity of *V. splendidus* I was also studied to know its potential to cause disease in larvae under captivity, as there was no report so far regarding *V. splendidus* I pathogenicity.

An attempt was also made to isolate the 'LB' from the intestinal tract of wild shrimp (*P. monodon* and *P. indicus*) and it was possible to retrieve maximum number of 'LB' from the GI tract. The discussion is presented in the following headings.

- Quantitative and qualitative distribution of 'LB' in the selective agar (SWC agar) from different larval stages of the hatchery cycle and from wild shrimp collected from fish landing of Cochin Fisheries harbour.
- Biochemical tests
- Antibiotic sensitivity
- β- Haemolytic activity
- Pathogenicity studies

In the present observation it was not possible to isolate 'LB' from different larval stages from the hatchery cycle, which showed good husbandry practices and proper chlorination of intake water (15 ppm) of the Vallarpadam hatchery. Karunasagar *et al.*, (1994) reported that filtered seawater intake was devoid of 'LB', they also added that the water treatment system of the hatchery (slow sand filteration and pressure sand filter) was capable of reducing 'LB'. Whereas Jawahar Abraham *et al.* (1997b) obtained 3.5 x 10^1 'LB' in sand filtered inshore well water. Karunasagar *et al.* (1994) reported that all zoea and PL harboured 10^2-10^6/gm of 'LB'. But in the present observation, zoea and PL of both *P. monodon* and *P. indicus* it was not possible to retrieve 'LB'. Lavilla pitogo *et al.* (1980) reported the presence of 'LB' in chlorinated and filtered seawater in the different localities in Panay Island, Philippines like Aklan Province (Numanica) and Capiz Province (pilar).

Ruby and Nealson (1978) studied nearshore surface water in southern California and found very predominant occurrence of *V. harveyi* during summer. O'Brien and Sizemore (1979) reported highest percentage of 'LB' in shrimp during summer. They also reported more number of 'LB' in shrimp when compared to water column. In the present study higher percentage of 'LB' occurred from wild shrimps collected from Cochin fisheries harbour. 33% of 'LB' was isolated from nearshore water of Cochin backwaters. Ruby *et al.* (1980) also

reported the occurrence of 30 CFU/100 ml of 'LB' in the spring and it was not detected in the winter along the surface waters of San Diego.

Based on the biochemical reactions the 'LB' isolated in the present study was identified as *V. harveyi* (38.46%), *V. splendidus* I (30.76%), *V. orientalis* (23.07%), and *P. leiognathi* (7.69%) (Table No. 6), based on the scheme of Alsina and Blanch (1994); West and Colwell (1984). Lavilla-pitogo *et al.* (1990): Karunasagar *et al.* (1994); Jawahar Abraham *et al.* (1999) followed the scheme of West and Colwell (1984) whereas Leano *et al.* (1998) followed the scheme of Alsina and Blanch (1994).

The aminoacid decarboxylase pattern observed in the present study was similar to that of *V.harveyi* associated with shell disease and mass mortalities in hatcheries by previous workers like Lavilla-Pitogo *et al.* (1990); Karunasagar *et al.* (1994); Jawahar Abraham *et al.* (1997b) which showed that the isolates obtained in the present work also may be opportunistic pathogens.

The intensity chart table No 6 indicates, the 'LB' composition as *V. harveyi* (38.46%), *V. splendidus* I (30.76%), *V. orientalis* (23.07%), *P. leiognathi* (7.69%). All 'LB' isolates were of halophilic in nature. Very predominant occurrence of *V. harveyi* was reported by Jayabalan *et al.* (1982) in the Vellar estuary, forming 81.25% of the total 'LB'.

TABLE 10 REPORTS OF THE PERCENTAGE COMPOSITION OF LUMINESCENT BACTERIA ISOLATED FROM DIFFERENT REGIONS

AUTHOR AND STUDY AREA SCHEME FOLLOWED	VIBRIO HARVEYI	VIBRIO SPLENDIDUS	VIBRIO ORIENTALIS	VIBRIO FISCHERI	PHOTOBACTERIU-M LEIOGNATHI
Jayabalan *et al.*, 1982 LB in sediments of Vellar estuary Reichelt and Baumann (1973)	81.25%			10.4%	8.3%
Jawahar Abraham *et al.*,1999 Tuticorin coastal waters	88.66%	3.35%	4.46%	1.86%	1.67%
Shrimp farm Environments West and Colwell (1984)	90.93%	2.11%	4.43%	0.42%	2.11%
Present Study 2000 Cochin Back waters Alsina and Blanch (1994) ;West and Colwell (1984)	38.46%	30.76%	23.07%		7.69%

In the Tuticorin coastal water *V. harveyi* remained predominant form of 'LB' forming 88.66% of total whereas in shrimp farm it was found higher than coastal water, the percentage being 90.93% of the total.(Table No.10) Out of 1,012 isolates of Tuticorin coast farm environment 5 different species were isolated eventhough six different species of 'LB' occurs in Indian waters (Jawahar Abraham *et al.*, 1999). In Japan eight different species of 'LB' has been reported by Venkateswaran *et al.* (1989). Organisms such as *V. logei* and *P. phosphoreum* were not encountered in Tuticorin as well as in Cochin as they are restricted to temperate region or deeper waters.

All the 13 cultures showed sensitivity to chloramphenicol (100%), streptomycin (100%), co-trimoxazole (84.61%) and nalidixic acid (84.61%). Multiple Drug Resistance (MDR) was very common for the drugs like tetracycline, ampicillin, gentamycin, penicillin and ciproflaxacin (Table No.8)

V. harveyi isolated in the present observation were sensitive to chloramphenicol, streptomycin, nalidixic acid and co-trimoxazole whereas Baticados *et al.* (1990) reported varied response of *V. harveyi* to chloramphenicol and low sensitivity to OTC. Karunasagar *et al.* (1994) recorded variants of *V.harveyi* isolated from larvae which were found resistant to streptomycin, chloramphenicol, co-trimoxazole. The antibiotic sensitivity pattern observed was entirely different from the antibiotic sensitivity of present isolates, which may be due to genetic

variation. The vibriostatic activity of these isolates towards 0/129 vibriostatic compound were also found to differ. Karunasagar *et al.* (1996) isolated *V. harveyi* from biofilm which was sensitive to chloramphenicol and tetracycline. Biofilm formation took place even in the presence of chlorine (15 ppm) which showed the isolates were harbouring enzyme potential to resist all the disinfectants or the slime serve as a protective layer for the flora. Pillai and Jayabalan (1996) also recorded multiple drug resistance of *V. harveyi* towards Ampicillin, Kannamycin, Penicillin, Streptomycin and Sulphadiazine.

V. splendidus I showed multiple drug resistance for Tetracycline, Ampicillin, Penicillin, Ciproflaxacin and even to Gentamycin which indicated the isolates of *V. harveyi.* and *V. splendidus* I were harbouring the R^+ plasmids in their extrachromosomal element. Baticados *et al.* (1990) reported varied response to chloramphenicol and prefuran and low sensitivity to OTC by *V. harveyi and V. splendidus* I. Multiple Drug Resistance (MDR) indicated the presence of R^+ factor with transferable drug resistance.

In the present study 61.53% of the isolates showed β-haemolytic activity which showed their high virulence. Liu *et al.* (1996b) found all the penaeid isolate of *V. harveyi* produces stronger protease, phospholipase and β-haemolytic activities than the reference strains (non-penaeid sources) suggested that all these factors may play a role in the pathogenicity of *V. harveyi* in tiger prawn (*P. monodon*) and kuruma prawn (*P. japonicus*). All the 12 *Vibrio harveyi* strains isolated

from snook cornea and aquarium water by Kraxberger *et al.* (1990) has shown 100% β-haemolytic activity of sheep RBC.

Pathogenicity studies:

V. harveyi:

60% mortality was observed at 10^5 cell/ml concentration in 48 hrs immersion experiments and luminescence was observed in water and larvae of *P. indicus*. Pillai and Jayabalan (1993) reported no sign of luminescence and mortality in their experiment conducted with *V.harveyi* towards *P. indicus* advanced PL in 10^3, 10^4 and 10^5 concentration but the re-isolation of the 'LB' was possible. Sae-Oui *et al.* (1987) recorded significant mortalities in 10^7, 10^8 CFU/ml when *V. harveyi* was challenged against *P. merguiensis*. Karunasagar *et al.* (1994) reported 80% mortality in *P. monodon* in immersion experiment with *V. harveyi* isolated from seawater, whereas 100% mortality was observed when infected with *V. harveyi* isolated from larval tank (in 5 day observation). In the present experiment 80% mortality within 48 hrs of immersion with *V. harveyi* (isolated from the gut of the wild shrimps) was recorded in *P. monodon*, whereas only 60% mortality was recorded in *P. indicus*. Lavilla-Pitogo *et al.* (1990) observed significant mortality in 10^2 and 10^3 cells/ml concentration within 48 hrs of immersion treatment of *V. harveyi* against *P. monodon* PL. Weak and moribund larvae became opaque and white especially in the thoracic region. The results of the present study resembled the above work. It

was possible to prove Koch's postulates in the present observation even though Koch's postulate with *Vibrio* sp. isolated from diseased shrimps have been unsuccessful, or successful only after challenge with a relatively massive inoculum, further supporting the argument that many bacterial infections (due most often to *Vibrio* spp) are due to infections by opportunistic pathogens that are part of their host's normal microflora (Lightner, 1988).

It was possible to re-isolate the 'LB' and characteristics of the isolates were found to be same of that used for immersion experiments.

***V. splendidus* I**

The present study revealed that *V. splendidus* I can cause considerable mortality even at a concentration of 10^4 cells/ml concentration. 20% mortality was recorded when it was challenged against *P. indicus* PL and 60% mortality was recorded in 10^5 cells/ml concentration (Table No.9)

In the case of *P. monodon* 60% mortality was recorded at 10^5 cells/ml concentration. As there is paucity of reports on *V. splendidus* I pathogenicity studies it is not possible to compare the results with other reports. Bacticados *et al.* (1990); Lavilla-pitogo *et al.*(1990); Karunasagar *et al.*(1994) reported that both *V. harveyi* and *V. splendidus* I are responsible for luminosis in hatcheries. The occurrence of significant mortality in shrimp *P. monodon and P. indicus* even at low concentration of both *V. harveyi* and *V. splendidus* I may be due to high

virulence of the selected strains. 80% mortality was recorded and the remaining PL were found healthy indicating that stress may have enhanced the suceptibility of the shrimps and 'LB' as opportunistic pathogen.

The source of infection of 'LB' may be from the resource water and it has been proved that luminescent bacteria is a natural flora of the GI tract of shrimps playing a role in causing luminosis whenever the animal is stressed in captivity as opportunistic pathogen.

Mortality in shrimp hatcheries may not be by luminous bacteria alone and it may be caused by several other environmental factors as well. The use of antibiotics can produce MDR strains which should be avoided. If healthy shrimps can be continually provided and maintained without stress, luminosis will not be a limiting factor for shrimp seed "Production".

6. SUMMARY

SUMMARY

1. The present study "Studies on Bionomics and Pathogenicity of Luminescent Bacteria" was carried out throughout the larval cycle of *P.monodon* and *P.indicus*. An attempt was also made to isolate 'LB' from G.I. tract of wild shrimp collected from Cochin fisheries harbour. The biochemical potential was studied in detail and pathogenicity studies in different concentrations of selected luminescent bacteria was done in *P. monodon* and *P. indicus* larvae which was found lethal in massive inoculum proving that *V. harveyi* and *V. splendidus* I can be reported as the etiological agent of luminosis.

2. Out of the two selective media used, sea water complex agar has been found best suited for isolation, maintenance and for re-isolation, to prove Koch's postulates. The luminescence was exhibited for a long duration in this agar.

3. In all wild shrimps and nearshore water 'LB' was detected but it was not possible to isolate 'LB' from hatchery larval stages.

4. Inoculation - re-isolation testing by Koch's postulates established the isolated organism like *V. harveyi* and *V. splendidus* I as etiological agent of luminosis.

5. Sea water complex agar was unable to support the growth of pigmented forms whereas in Nutrient agar various pigmented bacteria

were isolated from the same inoculum which indicated the selective potential of SWC agar, to support the isolates.

6. All 'LB' in the Seawater complex agar were cream coloured shiny, smooth, circular and raised colonies and developed with overnight incubation at RT. In the subculture, the luminescence started by 4 hours and gradually reached its maximum luminescence within 18 hrs and luminescence was retained upto 2-5 days in this agar.

7. Total 'LB' of nearshore water was found to be 5×10^4 CFU/ml and in wild shrimps *P. monodon* it ranged between 30×10^2 CFU/ml - 3×10^4 CFU/ml and in *P. indicus* 2×10^2 CFU/ml - 3×10^4 CFU/ml.

8. All reagents, ingredients and media used were from HI-MEDIA and standard methods were followed in isolation and identification procedures.

9. Catalase and oxidase were found positive for all isolates. Most of the isolates were inert to arginine dihydrolase whereas lysine and ornithine were decarboxylated by 61.5% and 38.5% respectively.

10. Glucose, sucrose, cellobiose were fermented by all 13 cultures, whereas lactose, arabinose, inositol were not fermented.

11. Salt-tolerance was highly variable with these isolates giving flourishing growth in 1.5 - 3.5% concentration of NaCl proving that all are halophilic in nature. No growth occurred in 10% NaCl concentration except one culture which showed trace growth.

12. Indole was produced by 92.30% of 'LB' whereas acetyl methyl carbinol production was found nil. Glucose was fermented by 12 isolates

and one was unable to ferment glucose in solid medium. Gelatin was hydrolysed by all the isolates which showed the high proteolytic potential of 'LB'.

13. Profuse growth was noted in TCBS by all isolates except one.

14. Sensitivity to antibiotics was demonstrated with HI-MEDIA discs 92.3% isolates showed multiple drug resistance (MDR) (Table 10) indicating the presence of R^+ factor with transferable drug-resistance.

15. 61.53% the 'LB' strain showed β - haemolysis

16. Pathogenicity studies by immersion experiments was found lethal in 10^5 cells/ml concentration. *V. harveyi* showed pathogenicity to both *P. indicus* and *P. monodon* PL at 10^5 cells/ml concentration. Whereas *V. splendidus* I isolated in the present study was pathogenic to *P. indicus* PL even at 10^4 cells/ml concentration and caused significant mortality in *P. monodon* at 10^5 cells/ml concentration. The above observations shows that both *V.harveyi* and *V. splendidus* I are capable of causing significant mortality under captivity.

17. The source of infection of 'LB' was from natural resource water and from the intestine of gravid females.

The studies on bionomics of 'LB' showed that by stress and improper management only the versatile luminescent heterotrophs are turning into opportunistic pathogens. The absence of 'LB' in Vallarpadam hatchery indicated good disinfection and husbandry care taken by hatchery operators.

If a good quality disease free gravid female shrimp can be continually provided and maintained in a stress and disease free facility, luminosis will not be a limiting factor in shrimp seed production.

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